## Remarks

Prior to entry of this amendment, claims 1-42 were pending in the application. Claims 8-9 and 31-32 are canceled herein. Applicants expressly reserve the right to pursue protection of any or all of the canceled subject matter in one or more continuing applications. Claims 1, 20 and 23 are amended herein. Claim 1 is amended to incorporate the limitations of claim 9. Claim 23 is amended herein to incorporate the limitations of claim 32. Support for the amendment of claim s 1 and 23 may be found throughout the specification. Support for the amendment of claim 20 can be found throughout the specification, such as on page 31, lines 16-21; page 32, lines 25-30; and page 34, line 18 to page 35, line 14.

New claims 43-47 are added herein. Support for new claims 43-47 can be found throughout the specification, such as on page 29, line 1 to page 32, line 5; page 21, lines 11-21, original claims 1, 3, 5 and 17; and page 35, lines 15-26, and in the original claims.

The specification is amended herein to properly refer to trademarks and correct typographical errors. Example 3 of the specification is amended herein to correct the reference to the figures, as supported in the specification on page 5, lines 11-30, Figs. 6-8, and page 35, line 15-26.

No new matter is introduced by the foregoing amendments or the new claim. After entry of this amendment, **claims 1-7, 10-30, and 33-47 are pending in this application**. Reconsideration of the pending claims is requested.

### *Restriction Requirement*

The election with traverse of the species of a) acute respiratory distress syndrome, b) inhalation, c) anti-inflammatory agent, and d) SEQ ID NO: 1 for initial prosecution was acknowledged in the Office action. Applicants expressly reserve the right to petition the restriction requirement.

### *Priority*

Applicants thank the Examiner for acknowledging the priority claim.

### *Specification*

The specification is amended herein to include trademarks in all capital letters. The specification is also amended herein to correct the reference to the figures, as supported on page 5, lines 11-30, Figs. 6-8, and page 35, line 15-26.

### *Rejections under 35 U.S.C. § 112, first paragraph*

Claims 1-41 were rejected under 35 U.S.C. § 112, first paragraph as allegedly the specification is not enabling for methods of treating a subject who is at risk for developing an inflammatory lung disease. Applicants respectfully disagree with this assertion.

The Office action alleges that the specification is only enabling for a method of treating inflammatory lung disease in a subject *using cells* that includes administering a therapeutically effective amount of a suppressive oligodeoxynucleotide (ODN). However, the Office action alleges that the specification is not enabling for (1) the claimed ODNs or (2) an *in vivo* method of treating inflammatory lung disease using a therapeutically effective amount of a suppressive ODN or. Applicants respectfully disagree with these assertions.

Applicants submit that one of skill in the art could readily make and use the claimed ODNs without undue experimentation. A Wands analysis is provided below:

(A) Breadth of the claims: The claims are limited to the treatment of an inflammatory lung disease using a suppressive ODN that is at least about 8 nucleotides in length, forms a G-tetrad, has a CD value of greater than about 2.9, and comprises at least two guanosines, wherein the oligodeoxynucleotide is from about 10 to about 30 nucleotides in length. Claims 17 and 41 recite specific nucleic acid sequences of use in the claimed methods.

(B) The nature of the invention: The claims are directed to methods of treatment that utilize nucleic acid sequences.

(C) The state of the prior art: The synthesis of ODNs is well known in the art, as is the administration of ODNs to a subject of interest. There are art-recognized models for testing therapies for the treatment of inflammatory lung disease.

(D) The level of ordinary skill in the art: The level of skill of a molecular biologist and/or pulmonologist is high.

(E) The level of predictability in the art: The production of ODNs with specified characteristics is routine. There are art recognized models for inflammatory lung disease (such as the one described in the examples section) that can be used to confirm the effect of an ODN of interest. The administration of agents to a subject for the treatment of inflammatory lung disease is described (see the specification, page 24, line 9 to page 30, line 10); the pharmaceutical formulations and methods of administration are routine to one of skill in the art.

(F) The amount of direction provided in the specification: The specification discloses the physical characteristics of ODNs of use, namely that they (1) are 10-30 nucleotides in length, (2) have a CD value of greater than about 2.9 (for example, see the specification on page 20, lines 15-24), and (3) form a G-tetrad (for example, see the specification on page 20, lines 7-14). General classes of ODNs that have these characteristics are provided (see the specification on page 21, lines 1-10), and at least 25 examples of suppressive ODNs are provided (see the specification on page 21, lines 11-21, and SEQ ID NOs: 1-25). In addition, pharmaceutical compositions and methods of administration of ODNs are described in the specification (see page 24, line 9 to page 30, line 10). Methods for confirming the effectiveness of an ODN, such as by monitoring lung function are disclosed (see the specification at page 30, line 11 to page 31, line 15).

(G) The existence of working examples: Numerous exemplary ODNs of use are described in the specification (see, for example, page 21, lines 15-21 and SEQ ID NOs: 1-25). The effectiveness of these ODNs in inhibiting an immune response *in vitro* is disclosed (see Example 2). The use of an exemplary ODN (SEQ ID NO: 1) in an animal model of inflammation is described in the specification (see Example 3). Thus, a working example is provided.

(H) The quantity of experimentation required: There is substantial guidance provided on the synthesis of ODNs and formulation of ODNs for delivery to a subject (see, for example, page 19, line 26 to page 24, line 7). The synthesis of ODNs is automated and routine. Thus, only limited experimentation is required to practice the claimed invention.

With regard to the use of suppressive ODNs, Applicants submit that the specification is fully enabling for methods for treating inflammatory lung disease, wherein the methods include the administration of a therapeutically effective amount of a suppressive ODN *in vivo*. The Office action states that the specification describes only general methods. The Office action further describes that Example 2 describes the use of suppressive ODNs to inhibit the secretion of cytokines from RAW 267.4 macrophage cells *in vitro*. Applicants agree that *in vitro* data is presented in Example 2, documenting the effect of suppressive ODNs on macrophage cells (which are important in the etiology of inflammatory lung disease).

However, additional data is presented in the specification. Example 3 demonstrates that suppressive ODNs block CpG-induced inflammation in an animal model, namely a mouse model of inflammatory lung disease. The Office action alleges that Example 3 utilizes RAW 267.4 cells, which is not correct. Page 33 of the specification describes the animal procedures:

> "Eight to twelve week old BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were maintained in an SPF facility. Animals were anesthetized with inhaled isoflurane, *and ODNs administered intratracheally using a sterile, blunt, fine bore needle*. At various times post treatment, animals were sacrificed. The trachea was exposed by a midline incision, an 18-gauge catheter inserted, and the lungs lavaged 4 times with 1 ml aliquots of PBS. The BAL fluid was pooled, centrifuged at 500 x g for 10 minutes at 4°C. Supernatants were stored at -70°C until assay. Cell pellets were resuspended in PBS, counted, spun onto a glass slide (Cytospin 2; Shandon, Pittsburg, PA), and individual cell types quantitated following staining with Diff-Quik (Dade Behring Inc., Newark, DE)." [emphasis added]

It is clear from this description that the ODNs are suspended in a carrier and administered to the animal intratracheally. Macrophage cells, such as RAW 267.4 cells, are **not** used at any time in this animal model.

The specification describes results obtained in this animal model in Example 3, page 35, line 15 to page 36, line 3 and Figs. 6-8. Specifically, it is disclosed that immunostimulatory CpG DNA instilled into the lungs of mice triggers inflammation. The administration of an exemplary inhibitory ODN, namely an ODN with a nucleic acid sequence set forth as SEQ ID NO: 1 (see the methods section page 32, line 17) resulted in a reduction in inflammation, as measured by the production of inflammatory cytokines and neutrophil infiltration (see page 35, lines 21-26 and Fig. 4). It is clear from the results presented in the specification that direct administration of a suppressive ODN, such as an ODN having the nucleotide sequence set forth as SEQ ID NO: 1, can be used to treat inflammatory lung disease.

The Office action cites several references, such as Goldberg et al. (Immunol. Lett. 73: 13-18, 2000, copy not provided to the Applicant). Goldberg et al. describes immunostimulatory CpG ODN and their role in disease. The Office action indicates that Goldberg et al. state that ODNs merit further study. Applicants do not deny that in 2000 Goldberg suggested that immunostimulatory ODNs should be studied to determine their role in disease. However, the present application describes the use of immunosuppressive ODNs, NOT immunostimulatory ODNs. Thus, the ODNs described in the present application are *completely different* than the ODNs discussed in Goldberg et al. Moreover, the statements made in Goldberg et al. (albeit in 2000, two years before the priority date of the present application), suggest that *ODNs could be of use*. It is the work of the Applicants that defines both the sequence of the specific ODNs of use (namely that they are not unmethylated CpG ODNs but are of 10-30 nucleotides in length with a defined CD value and form a G-tetrad) and the utility (in treating inflammatory lung disease) of the claimed ODNs.

The Office action further refers to Heeg et al., International Archives of Allergy and Immunology, page 93, 2000. Heeg et al. states that immunostimulatory CpG ODNs have been used as vaccine adjuvants and selectively induce a Th1 response, and suggest that these ODNs could be used for the treatment of allergy. The Office action alleges that Heeg et al. question whether experiments performed in mice will be successful in humans. However, the quotation cited in the Office action states "the data so far indicate that human cells respond in a similar way to murine cells." Thus, although Heeg et al. describe the effects of completely different ODNs (those that are immunostimulatory), it appears that Heeg et al. conclude that human cells will respond to ODNs in a manner similar to murine cells, and suggests that ODNs could be used in immunotherapy (see the last sentence of the abstract). Applicants submit that, contrary to the negative statements made in the Office action, Heeg et al. supports the assertion that a description that sets forth specific ODN sequences, and provides data in an animal model that these ODNs are effective, provides enablement for the treatment of humans.

Broide et al. (J. Immunol. 161: 7054-7062, 1998) describes that the mouse model of allergen induced hyperresponsiveness to demonstrate that an immunostimulatory ODN with a CpG motif (again, not the inhibitory ODNs of the present application) can be used to inhibit airway eosinophila and reduce responsiveness to the triggering agent. Briode et al. state (page 7062) "In summary, ISS-ODN [immunostimulatory ODN] administration provides an alternative to the current practice of allergen protein desensitization, which has relative low efficacy and high potential for side effects, such as anaphylaxis..." The Office action cites this reference as demonstrating that there are limitations on anti-inflammatory agents for the treatment of inflammatory lung disease. However, Broide et al. are commenting on the limitations of allergen protein desensitization, NOT on the limitations of ODN therapy. Any negative statements made by Broide et al. four years prior to the priority date of the present application with regard to other treatments such as allergen protein desensitization, and the suggestion that ODNs should be further studied, simply cannot negatively reflect on the present disclosure. The Applicants clearly have demonstrated that the immunosuppressive ODNs can be used to treat inflammatory lung disease, and have described these methods in sufficient detail for one of skill in the art to make and use the claimed invention.

The Office action further refers to Bernard et al. (Amer. J. Resp. Crit. Care Med. 72: 798-806, 2005) as describing that corticosteroids are a standard therapy for inflammatory lung disease. The Office action appears to allege that this post-filing date reference suggests that treatment for ARDS simply is not possible. However, this is in stark contrast to the conclusions of Bernard et al., who begin by stating "....the advances made even in the past 50 years are staggering when compared with all else that came before..." (see page 798). Bernard et al. do describe the use of corticosteroids as controversial (see page 803). However, Bernard et al. suggest that there is considerable promising work, such as from the NHLBI using modern techniques such as microarrays and proteomics. Indeed, Bernard et al. summarizes how effective new techniques could be on page 804:

> "The past 100 years (especially the last 50 years) of ARDS research and advances in the clinical care of patients with ARDS is a fascinating picture that spans the timeframe of so many other major medical and scientific advances. Tools such as the highly instrumented animal model, cell biology, molecular biology, advances in imaging from the micro to macroscopic, and genetics have greatly facilitated

> our understanding of this complex syndrome. The scientific foundation is strong and will provide a firm basis upon which the next chapter in this saga can be written. With only a little imagination, a day can be seen when the mortality and the long term morbidity of ARDS will be reduced to only that of the preexisting underlying diseases, if any."

Thus, Bernard et al. simply cannot be viewed as providing evidence that inflammatory lung disease cannot be treated by any method. Contrary to the negative assertions in the Office action, Bernard et al. actually suggest that new strategies for the treatment of inflammatory lung disease could be very effective. Thus, Bernard et al. does not negate the results presented in the specification.

The Office action states that the "murine model is generally the experimental animal used in studies for inflammatory lung disease or lung injury. Animal models of lung injury have been useful in the mechanisms in inflammatory lung disease. However, using an animal model that mimics key aspects of human inflammatory lung disease has been limited." In support of this statement, the Office action cites Matthay et al., (American J. Resp. Crit. Care Med 167: 1027-1035, 2003). This article describes the recommended future research directions for acute lung injury suggested by the NHLBI working group. This article states (page 1032) "Animal studies that have attempted to mimic human ALI/ARDS have been useful and will likely continue to provide valuable observations regarding both the mechanisms underlying the pathogenesis, progression and resolution of this syndrome and ways in which its course can be modulated therapeutically." Although Matthay et al. discloses that the there is uncertainty about which of the many available animal models most accurately reflects the human clinical syndrome, this article suggests that the study of animal models can be used to evaluate "the effect of agents having the potential to diminish the severity of systemic and pulmonary injury…" (see the sentence bridging pages 1032-3). Thus, contrary to the negative conclusions asserted in the Office action, Matthay et al. also support the conclusion that treatments can be demonstrated to be of use in animal models (such as the one described in the examples section of the present application.

The Office action further cites van der Vliet (Resp. Res. 1: 67-72, 2000) as describing shortcomings of several animal models of respiratory tract disease. Applicants do not deny that every animal model does not replicate every aspect of a human disease process. However, this does not negate the overall utility of animal studies. Submitted herewith as Exhibit A is Dawkins and Stockley ("Animal models of chronic obstructive pulmonary disease," Thorax 56: 972-977, 2001), which describes the usefulness of animal models of respiratory disease. For example (see page 972, first column):

> Animal models act as a bridge between in vitro studies in the laboratory and studies of humans. As such, they have had a major impact on the investigation of many medical conditions...animal models can provide a framework for the rational and safe design of expensive and long term clinical studies.

This article concludes (page 976):

> Rodents and humans have certain anatomical and physiological differences in the broncopulmonary trees but mount similar host responses to lung challenge....Such approaches will not only increase our understanding about pathogenesis, but will also facilitate the development and introduction of new therapeutic strategies.

Animal models are known to be of use for studying acute lung injury. For example, Metz and Sibbald (Chest 100: 1110-9, 1991, copy submitted as Exhibit B) states:

> A wide variety of animal models have been developed to explore both the pulmonary and nonpulmonary sequelae of acute lung injury. Common to a majority of these models has been the description of a diffuse inflammatory reaction in the lung's microvasculature....recent data from animal studies of anti-inflammatory therapy in ALI have generated interest in clinical trials of anti-inflammatory therapy for ARDS.

Thus, one of skill in the art is not "accepting on its face that the examples given in the specification as being correlative or representative of the successful treatment or inhibition of inflammatory lung disease in any subject (human or otherwise)" (see the Office action, page 11). Rather, one of skill in the art would evaluate the data presented in an **art-accepted** animal model of inflammatory lung disease, and agree that the Applicants have clearly enabled the treatment of inflammatory lung disease (as the data presented document that the claimed ODNs are efficacious).

The MPEP § 2107.03 states:

> As a general matter, evidence of pharmacological or other biological activity of a compound will be relevant to an asserted therapeutic use if there is a reasonable correlation between the activity in question and the asserted utility. *Cross v.*

> *Iizuka*, 753 F.2d 1040, 224 USPQ 739 (Fed. Cir. 1985); *In re Jolles*, 628 F.2d 1322, 206 USPQ 885 (CCPA 1980); *Nelson v. Bowler*, 626 F.2d 853, 206 USPQ 881 (CCPA 1980).... If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process.... Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials. There is no decisional law that requires an applicant to provide data from human clinical trials to establish utility for an invention related to treatment of human disorders (see *In re Isaacs*, 347 F.2d 889, 146 USPQ 193 (CCPA 1963); *In re Langer*, 503 F.2d 1380, 183 USPQ 288 (CCPA 1974))."

In the present application, the *in vitro* and *in vivo* results both document the activity of the claimed ODNs for the treatment of inflammatory lung disease. MPEP § 2164.02 states:

> An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention....In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate.

Working examples are provided both in vitro (RAW 264.7 cells, Example 2) and in vivo (mouse model of lung inflammation, Example 3). The MPEP clearly states that results from an art-accepted an model (and *in vitro* data) should be accepted as a showing that the claimed invention is enabled. Reconsideration and withdrawal of the rejection is respectfully requested.

Claim 1 is rejected under 35 U.S.C. § 112, first paragraph as allegedly not complying with the written description requirement. The Office action alleges that there is insufficient written description is provided for an ODN of "at least about 8 nucleotide in length" that "forms a G tetrad" and "has at least two guanosines." Applicants respectfully disagree with this rejection. Applicants note only claim 1 was included in the rejection. Thus, it is the Applicants understanding that the U.S. PTO considers there to be sufficient written description for the remaining claims. Solely to advance prosecution, claim 1 is amended herein to incorporate the limitations of claim 9, and thus to be limited to nucleotides of 10 to 30 nucleotides in length. Applicants submit that this amendment overcomes the rejection.

With regard to written description: The specification discloses the physical characteristics of ODNs of use, namely that they (1) are 10-30 nucleotides in length, (2) have a CD value of greater than about 2.9 (for example, see the specification on page 20, lines15-24), and (3) form a G-tetrad (for example, see the specification on page 20, lines 7-14). General classes of ODNs that have these characteristics are provided (see the specification on page 21, lines 1-10), and at least 25 examples of suppressive ODNs are provided (see the specification on page 21, lines 11-21, and SEQ ID NOs: 1-25). In addition, pharmaceutical compositions and methods of administration of ODNs are described in the specification (see page 24, line 9 to page 30, line 10). Methods for confirming the effectiveness of an ODN, such as by monitoring lung function are disclosed (see the specification at page 30, line 11 to page 31, line 15).

The Office action (see page 14) cites the Guidelines for Examination of Patent Applications under 35 U.S.C. § 112, paragraph 1 as stating "possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing the invention was 'ready for patenting' such as by disclosure of drawings or structural chemical formulas that show the invention was complete, by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention." Applicants would like to draw the Examiner's attention to a drawing (Figure 1A), which illustrates the specific structure of an oligodeoxynucleotide that forms a G tetrad. As described in the specification, a G tetrad involves the planar association of guanosines in a cyclic Hoogsteen hydrogen bond arrangement. The specification provides information on specific physical and stereochemical properties of these structures (see, for example, page 20). G-tertrads can be classified based on the circular dichromotism (CD) values. Specific lengths of ODNs of use are provided. In addition, a specific subclass of ODNs of use is described: ODNs with TTAGGG repeats. Moreover, 25 specific examples of ODNs of use are provided as SEQ ID NOs: 1-25 (see page 21).

An applicant seeking to claim a genus of compounds, such as a nucleic acid must provide a "representative" number of species within the genus. It is well established that an Applicant need not disclose every species encompassed by a claim (In re *Angstadt*, 537 F.2d 498, 190 USPQ 214 (CCPA 1976). In the present application, ODNs with specific physical and stereochemical properties are disclosed. The structure of these molecules is clearly set forth. In addition, 25 examples are provided. Thus, there clearly is sufficient written description for the claimed ODNs.

Reconsideration and withdrawal of the rejection is respectfully requested.

### *Rejection under 35 U.S.C. § 112, Second Paragraph*

Claim 20 was rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for not reciting either in vitro or in vivo. Claim 20 is amended herein to indicate that the cell is in vitro, thereby rendering the rejection moot.

### *Information Disclosure Statements: Claims Free of the Prior Art of record*

Two Information Disclosure Statements were submitted for the present application, one dated October 7, 2003, and one dated March 31, 2005. For the Examiner's convenience, copies of the PTO-1449 forms are included with this response. Applicants respectfully request that the Examiner initial and date the PTO-1449 to indicate that the cited prior art has been considered.

The Office action has cited U.S. Patent No. 6,214,806 (hereinafter the '806 patent) as being pertinent, but has not asserted any rejections based on the '806 patent. Thus, it is the Applicants understanding that all of the pending claims were free of the prior art.

### *Request for a PTO-892*

Applicants respectfully request a PTO-892 form be provided to make the references cited in the Office action formally of record in the present application.

### *SEQ ID NO: 1*

Applicants elected the species of SEQ ID NO: 1 for initial prosecution. No rejections based on any prior art were asserted in the Office action. Thus, it is the Applicants understanding that an oligodeoxynucleotide comprising the nucleic acid sequence set forth as SEQ ID NO: 1 is free of the prior art of record.

Data is presented in the specification (see Examples 2 and 3) documenting the effectiveness of an oligodeoxynucleotide comprising the nucleic acid sequence set forth as SEQ ID NO: 1 when used in models (in vivo and in vitro) of inflammatory lung disease. Data is also presented documenting the effect of oligodeoxynucleotides comprising the nucleic acid sequence set forth as SEQ ID NO: 1 on cytokine production (see the examples). Thus, it is clear that the specification is enabling for methods of treating inflammatory lung disease and reducing cytokine production in a subject that include administering to a subject a therapeutically effective amount of an oligodeoxynucleotide comprising the nucleotide sequence set forth as SEQ ID NO: 1. Allowance of new claims 43-47 is respectfully requested.

## Request for Interview

If an additional rejection is asserted, or if the present rejections are maintained, the Examiner is formally requested to contact the undersigned prior to issuance of the next Office action, in order to arrange a telephonic interview. It is believed that a brief discussion of the merits of the present application may expedite prosecution. This request is being submitted under MPEP §713.01, which indicates that an interview may be arranged in advance by a written request.

## Conclusion

It is respectfully submitted that the present claims are in a condition for allowance. Should the Examiner have further questions or comments with respect to examination of this case, it is respectfully requested that the Examiner telephone the undersigned so that further examination of this application can be expedited.

Respectfully submitted,

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By ______________________________
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*Thorax* 2001;56:972–977

## *Occasional review*

# Animal models of chronic obstructive pulmonary disease

P A Dawkins, R A Stockley

Chronic obstructive pulmonary disease (COPD) is the fifth leading cause of death in the UK,[1] causing 26 000 deaths and 240 000 hospital admissions and costing the NHS £486 million per annum. It is also a global problem, reaching almost epidemic proportions in the developing world. Despite this, it receives relatively little attention compared with diseases with a similar impact such as coronary heart disease and cancer. The research into new treatments to prevent the development and progression of this condition presents a major challenge. It is thus important to develop tools that can both direct development of new drugs and provide insights into assessment of their efficacy before extensive human trials are undertaken.

Animal models act as a bridge between in vitro studies in the laboratory and studies in humans. As such, they have had a major impact on the investigation of many medical conditions. In COPD, in particular, animal models would enable basic research to investigate the mechanisms of inflammatory cell recruitment and abnormal matrix repair (the proteinase/antiproteinase hypothesis[2]) and alternative hypotheses of the pathogenesis of emphysema such as those implicating lung cell apoptosis as a primary event.[3] They would also facilitate the testing of new treatments such as gene therapy (in cystic fibrosis and $\alpha_1$-antitrypsin deficiency (A1AT)) and "designer drugs" targeting specific cytokines or biochemical pathways. By leading to a clearer understanding of the key events in the pathophysiology of COPD and enabling short term studies to develop appropriate strategies, animal models can provide a framework for the rational and safe design of expensive and long term clinical studies.

### Advantages and disadvantages of mouse models

The mouse provides the best choice for an animal model[4] because the mouse genome has been extensively studied and sequenced and close similarities exist with the human genome. In addition, complementary antibodies and probes exist to many mouse enzymes, enabling them to be studied directly for quantification and localisation. There is also a rapid reproductive turnover, with large litter sizes and a short lifespan enabling studies to be completed within months. Breeding, housing, and maintenance costs are relatively low.

However, animal models have a number of limitations that have to be borne in mind. There are certain anatomical and physiological differences between the respiratory tract of mice and humans.[5] For instance, in mice there are no extensive cilia, few submucosal glands in the trachea, and no goblet cells. Mice do not expectorate sputum, are obligate nasal breathers that filter tobacco smoke inefficiently, and have less branching of the bronchial tree without respiratory bronchioles. The profile of inflammatory mediators is also slightly different in the mouse. For instance, the interstitial collagenase MMP-1 is found in humans but not mice[6] and, although mice have receptors for important pro-inflammatory cytokines found in humans such as interleukin (IL)-8[7] and leukotriene B (LTB)-4,[8 9] their role in mice has not been ascertained. Extrapolations of findings from the mouse to the human therefore have to be made with these differences in mind.

### First animal models: exposures

The first animal models of COPD stemmed from the extrinsic exposure to inflammatory stresses. Gross *et al* in 1965 described the first reproducible model of emphysema by instilling papain (a plant protease) into the lungs of rats.[10] Together with the observation of Laurell and Eriksson two years earlier of an association between serum A1AT (a protease inhibitor) deficiency and chronic airways disease and emphysema,[11] this animal model provided the basis of the proteinase-antiproteinase hypothesis of emphysema. Various proteases have since been introduced into the lungs of laboratory animals. However, these are relatively crude models in which the lung injury is caused by a single massive insult rather than a continuous low grade inflammatory process (which is believed to underlie smoking related emphysema). Also, the delivery of proteinases into the airways by a bolus in solution may not reflect the processes resulting from delivery of the enzymes in quantum packets by viable inflammatory cells to the interstitium in human disease.[12] Nevertheless, such models can be useful for studying events after the insult, as in the study of alveolar repair in response to treatment with a retinoic acid derivative[13] following instillation of porcine pancreatic elastase to create the initial emphysema model in rats.

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Received 27 July 2001
Accepted for publication
30 July 2001

EXHIBIT A

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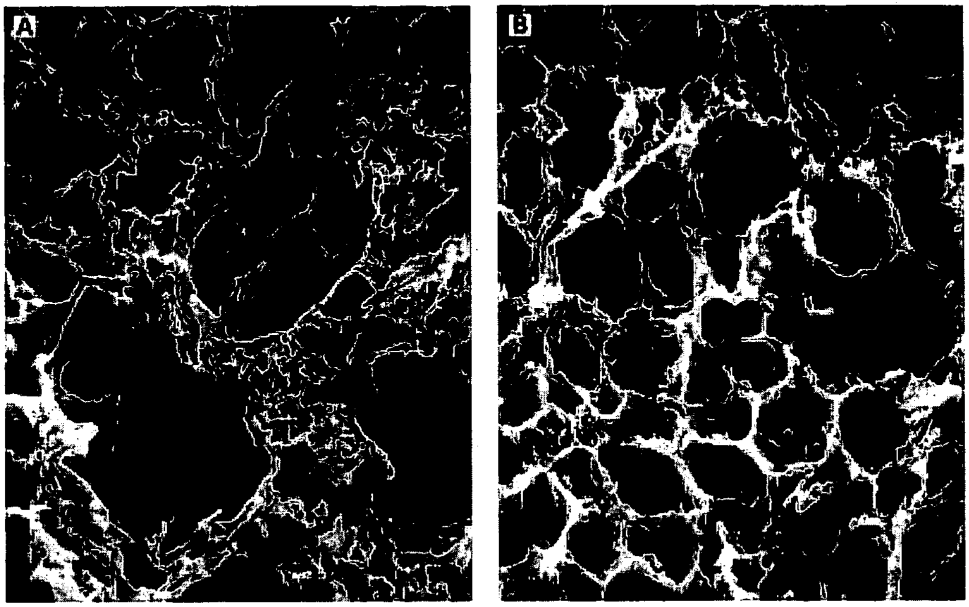

*Figure 1 Effect of cigarette smoke exposure on airspaces of C57BL/6 mice. Scanning electron microscopy ×400 of (A) lungs of mice exposed to cigarette smoke for 6 months and (B) lungs of non-exposed age-matched controls. Reprinted from Shapiro with permission of the publishers.*[17]

There are several tobacco smoking animal models of emphysema. Certain laboratory strains of mice are more susceptible to the effects of cigarette smoke—for example, C57BL/6, fig 1—and rats are less susceptible than mice. Dhami *et al*[14] used C57BL/6 mice exposed to the smoke of two cigarettes using a standard smoking apparatus to demonstrate that the administration of human A1AT or antineutrophil antibodies prevented the early breakdown of elastin and collagen, supporting the role of neutrophil elastase in acute lung damage due to cigarette smoke. However, in other smoking models the accumulation of macrophages has also been shown to be a feature necessary for producing some of the long term pathological features of emphysema.[15]

In order to create a model of A1AT deficiency, D-galactosamine has been administered intraperitoneally to rats,[16] resulting in decreased circulating A1AT levels and a propensity for more severe emphysema after the intravenous injection of pancreatic elastase. The emphysema was argued to have been caused by a proteinase/antiproteinase imbalance, but other glycoproteins that are also affected by D-galactosamine may have influenced the development of emphysema.

### Models resulting from natural mutations

Some naturally occurring mutant strains of C57BL/6 mice are more prone to developing emphysema including *tight skin*, *pallid* and *blotchy* variants. They tend to have lower serum concentrations of A1AT but this alone is not thought to be responsible for the development of emphysema. The airspace enlargement probably results from abnormalities in lung development rather than destruction of mature lung tissue. *Tight skin* mice[17] have a mutation of the fibrillin-1 gene that affects formation of elastic fibres which are central to normal alveolar development. *Pallid* mice[18] have a mutation affecting syntaxin 13[19] (a cell membrane protein) that gives rise to gradual development and progression of emphysema. *Blotchy* mice[20] have abnormal translation of the Menkes gene on the X chromosome and develop a connective tissue disorder similar to the human cutis laxa with associated abnormal lung matrix. These models may provide further insight into the importance and dynamics of normal lung matrix formation, damage, and repair. Their role in determining the pathogenesis and treatment of human emphysema remains unknown, although cutis laxa is associated with the development of emphysema in man.[21]

### "Knockout" mouse models

"Knockout" mouse models have been developed to disrupt the expression of a targeted gene as a result of the insertion of another gene or nucleotide sequence, hence they are often called "loss of function" models.

They are created by the process of targeted mutagenesis.[22] This relies on a "targeting" construct that has flanking sequences that match those in the gene of interest and contains markers for positive and negative selection, such as the phosphoglycerate kinase/bacterial neomycin resistance gene (*PGK-neo*) and phosphoglycerate kinase/viral thymidine kinase gene (*PGK-tk*), respectively. The construct is microinjected into embryonic stem (ES) cells obtained from the inner cell mass of a blastocyst, which have the potential to differentiate into any tissue in the body. "Homologous recombination" then occurs resulting in exchange of the artificial DNA sequence for the corresponding area of genomic DNA as the DNA breaks and rejoins. The probability of this happening is increased by the presence of the matching sequences that flank the target gene. The ES cells (from black mice) that have

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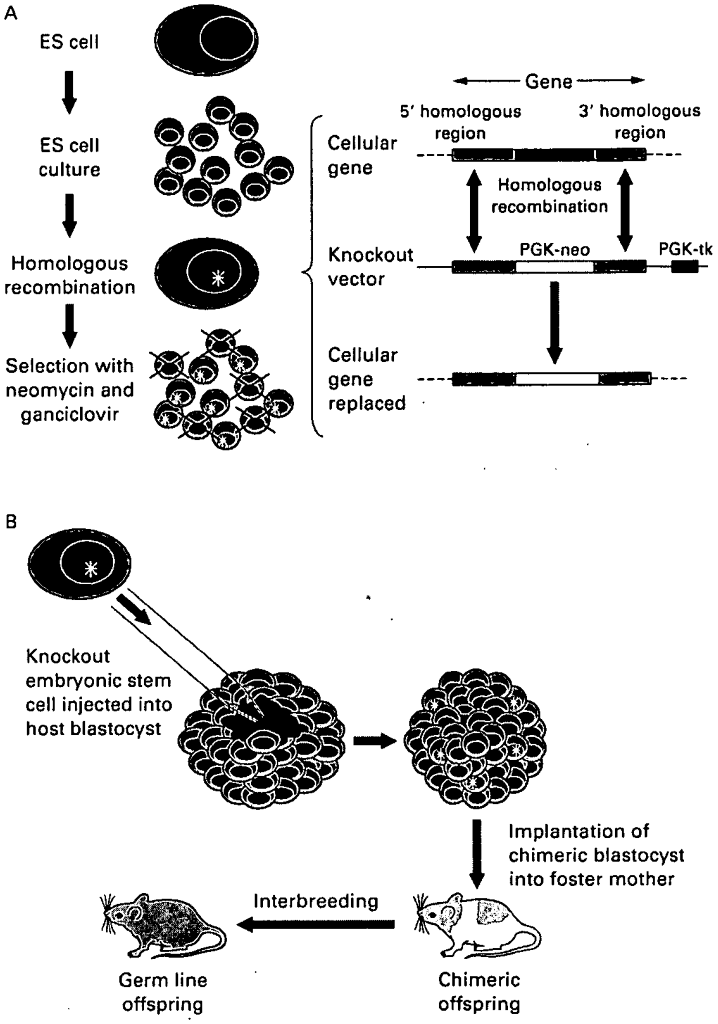

*Figure 2 Method of targeted mutagenesis (based on Majzoub and Muglia[22]). (A) A knockout vector is created consisting of the phosphoglycerate kinase/bacterial neomycin resistance gene (PGK-neo) flanked by segments homologous to the cellular gene and the phosphoglycerate kinase/viral thymidine kinase gene (PGK-tk) downstream. The vector is introduced into embryonic stem (ES) cell culture from black mice. Homologous recombination occurs between the vector and the cellular gene at the matching flanking regions, resulting in incorporation of the "knockout" vector, including the PGK-neo but not the PGK-tk, into the genome of the ES cell. The presence of the PGK-neo and the absence of PGK-tk allow survival of these ES cells after positive/negative selection with neomycin and ganciclovir. (B) The mutant clone of ES cells is then microinjected into the host blastocyst (from a white mouse) which is implanted into a foster mother resulting in the development of a chimeric mouse. The incorporation of the ES cells into the germ line of the chimeric mouse results in heterozygous germ line transmission of the mutant gene. The heterozygotes are then mated to produce offspring homozygous for the mutant gene, identified by reversion to the black phenotype.*

correctly recombined with the construct are selected out using neomycin and ganciclovir (due to the presence of the *PGK-neo* gene and absence of the *PGK-tk* gene). These are then injected into the host mouse blastocyst (from white mice) and impregnated into a foster mother. The result is a chimeric mouse (black and white). Chimeras that incorporate the ES cells in their germ line (for instance, the sperm), when bred with white mice will produce offspring heterozygous for the gene deletion (speckled or "Agouti" mice), and interbreeding of these in turn will lead to homozygous offspring (black mice) for the required mutation. The targeted mutation can thus be followed by coat colour (fig 2).

This strategy, if successful, results in mice that develop in utero with a single gene deletion. The major problem with this approach is that the gene defect may also affect prenatal and postnatal development leading to changes that may be relevant to disease expression in later life. Alternatively, there may be a degree of overlapping function in metabolic processes so that normal development is protected against the loss of the gene (redundancy). However, absence of one gene during development may lead to upregulation of components in compensatory pathways, thereby complicating the final phenotype of the "knockout" mouse.

Ideally, therefore, "knockout" animals should be allowed to develop normally before the gene of interest is deleted. This can be achieved with the Cre-lox P technology[23] which involves the insertion of specific nucleotide sequences called lox P either side of the target gene. The Cre enzyme (Cre recombinase, derived from a bacteriophage P1 that infects *Escherichia coli*) recognises the sequences flanked by the lox P sequences and causes DNA cleavage at these points, thereby deleting the gene. The advantage of this system is that relatively large sections of DNA can be excised after transfection of ES cells with Cre. Also, the Cre gene itself can be inserted into the mouse genome and modified so that it can be expressed only in specific tissue by being linked to a cell specific promoter. When this mouse is crossed with a mouse that has a target gene flanked by lox P sequences, this gene will only be "knocked out" in those specific tissue cells. The Cre gene can also be made to be inducible by using a cell specific promoter that can be activated by the presence of another molecule. For instance, promoter Mx1 in T cells can be induced by interferon (IFN)α leading to the expression of the linked DNA-polymerase-β gene.[24] Gene deletion can therefore be controlled in time and place by administration of the inducing agent (such as IFNα) to the mice leading to the controlled expression of Cre and hence cleavage of the target gene again only in the cells of interest. These sophisticated techniques remove the complicating issues of possible interference with developmental processes by "knocking out" the gene after development has occurred, and lack of tissue specificity by only deleting the product in cells of interest (fig 3).

"Knockout" models for emphysema include those for platelet derived growth factor A[25] which lose myofibroblasts and therefore have decreased elastin deposition, and the double "knockout" mutants for fibroblast growth factor receptors 3 and 4[26] which exhibit abnormal alveolar formation and septation. It is also noteworthy that both macrophage elastase[15] and neutrophil elastase "knockout" mice[27] are protected from tobacco smoke induced emphysema, supporting the role of these genes in the pathogenesis of emphysema.

Cystic fibrosis is caused by a single gene defect and is a common disease affecting a young population. As a result of an extensive research effort, several "knockout" mouse

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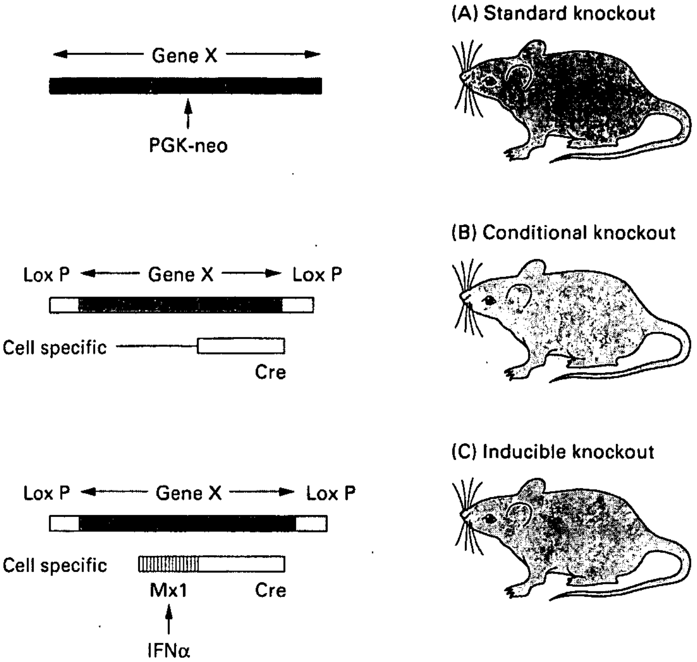

*Figure 3 Strategies for creating "knockout" mice (after Shapiro[1]). (A) The standard knockout is formed from the incorporation of the neomycin resistance gene driven by the phosphoglycerate promoter (PGK-neo) into gene X. This mutation introduced into embryonic stem cells renders this gene inactive throughout development and adult life. (B) Conditional knockouts achieve cell specific gene deletion by flanking gene X with lox P sequences. When these mice are crossed with a transgenic mouse expressing Cre recombinase under cell specific control, the gene X is only excised in those cells expressing Cre. (C) Inducible knockouts use the same strategy as conditional knockouts but the Cre gene is driven by a promoter that only allows transcription in the presence of the inducing agent. In this example the interferon (IFN)α inducible promoter Mx1 is shown.[22] IFNα can be introduced when gene knockout is specifically required.*

models for this condition exist.[28-31] These have been important in the study of the mechanisms of lung and intestinal disease in this condition, as well as potentially providing a system for the development and testing of treatments for patients with cystic fibrosis.

## Transgenic mouse models

Transgenic mice have genes of interest inserted into their genome with the resultant expression or overexpression of the relevant product, and hence are often called "gain of function" models. Various transgenes have given rise to the development of emphysema in mice, often discovered serendipitously. The metalloproteinase-1 (interstitial collagenase) transgene linked with the haptoglobin promoter unexpectedly caused emphysema as a result of expression in the lung.[32] Again, whether this was due to an effect on lung development or due to destruction of mature lung connective tissue is unknown. Transgenic mice overexpressing platelet-derived growth factor B, driven by the lung specific surfactant protein C promoter, also developed emphysema,[33] but this was in combination with fibrosis and therefore may have been due to tethering of the airways which is a process analogous to the focal emphysema of coal workers' pneumoconiosis.

The *klotho* mouse[34] was originally intended to be a model for hypertension as a consequence of the insertion of a sodium channel transgene leading to hypernatraemia. This primary aim failed, but the animals were observed to exhibit signs of premature ageing and airspace enlargement. It was found that the transgene had disrupted the *klotho* gene which codes for a transmembrane protein. It is uncertain whether the lungs ever develop fully in these mice before emphysema occurs, so its role in the study of the human ageing process in the lungs is uncertain.

The disadvantage with transgenic methods in elucidating the role of a gene product in the aetiology of adult emphysema is that the gene of interest is also expressed throughout organ development and growth, rather than at the preferred time after maturation. This problem led to the development of the technique of inducible transgenic expression. Zheng and colleagues[35] targeted overexpression of IL-13—a T helper 2 (Th2) pathway cytokine usually associated with atopy and asthma—to the lungs of mice. The authors used two constructs that included the Clara cell promoter CC10 which targets expression to the lung, and the reverse tetracycline transactivator (rtTA) which allows IL-13 gene transcription only in the presence of tetracycline given in the diet when required. They found that these mice develop emphysema, mucous metaplasia, and inflammation which are metalloproteinase and cysteine proteinase dependent. Similar but more gradual pathological changes occurred using the same method of inducible overexpression of IFNγ,[36] a T helper 1 (Th1) pathway cytokine associated with inflammatory reaction to infection in the lungs. These two models suggest that the pathogenesis of emphysema is complex, implicating both Th1 and Th2 pathways.

The question of development dependent and independent phenotypic expression has been addressed by the same CC10-rtTA method in a study on the effect of a transgene for IL-11 which caused emphysema in mice when constitutively expressed but not when inducibly expressed after lung development.[37] The results suggested that IL-11 exerts its effect during lung development, although the mechanism remains unknown.

## The next generation: "knock out"/"knock in" mice

Following the "knock out" of murine genes, the technology is being developed to insert (or "knock in") equivalent human genes in their place. In relation to pulmonary medicine, this is of major relevance in A1AT deficiency where the human variants (M, Z and S) can be inserted into the mouse genome after the murine A1AT gene has been "knocked out".

In mice there is known to be a cluster of four or five A1AT-like genes, depending on the strain, on chromosome 12 (S Shapiro, N Kalsheker, personal communication). This is different from humans where there is just one functional gene[38] and one pseudogene of unknown importance[39] on chromosome 14. Elucidation of the base sequence of the A1AT gene cluster in the mouse followed by the design of specific constructs will enable the

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"knock out" of the mouse gene locus and the subsequent "knock in" of the human equivalent. This will generate a more accurate model of A1AT deficiency than the straightforward "knockout" (which is the equivalent of a null mutation) since the mutant human genes will be expressed and the abnormal human A1AT protein will be produced and hence "tolerated" during development.

The model could then be used to research the pathogenesis of emphysema and bronchial disease in A1AT deficiency more accurately, since it should represent the human equivalent of reduced secretion of the abnormal protein and intracellular accumulation due to polymerisation.[40] The mice could then be exposed to a variety of relevant inflammatory stimuli—including cigarette smoke, elastase, cytokines, infections or endotoxin—to study their relevance in the development of human disease. In addition, the mice could be used to test treatments such as A1AT augmentation since repeated doses of human A1AT could be given without the development of an immune response. The standard "knockout" mice are likely to react to the "foreign" human epitopes on the second and subsequent administrations, leading to the development of antibodies that prevent the efficacy of repeated treatments. These models could also be used to test the efficacy of gene therapies with adenoviral[41] or adenovirus associated[42] vectors that deliver the genetic information for producing protective proteins such as A1AT to the lung. Other treatments that could be tested using these models include peptides to block intracellular A1AT polymerisation of the abnormal Z protein,[40] drugs that increase cellular secretion of A1AT,[43] and drugs that inhibit elastase production or secretion.[44]

It is possible to develop further models by crossing A1AT deficient mice with other human A1AT variants to study the effect (if any) of the heterozygous state. In addition, cross breeding with neutrophil elastase "knockout" mice or neutrophil deficient mice will lead to further understanding of the interrelationship of these key antagonistic elements in the development of disease. It would enable the proteinase/antiproteinase hypothesis to be confirmed if the double mutants of the key pathogenetic components revert to the same susceptibility to emphysema as the wild-type mice.

## Conclusion

Rodents and humans have certain anatomical and physiological differences in their bronchopulmonary trees but mount similar host responses to lung challenge. Animal models of COPD have evolved from the rather crude one-hit elastase exposure models to the sophisticated mutant strains being created by techniques at the cutting edge of genetic science. These advances enable us to enter a new era of scientific research into COPD, where individual mediators and their abrogation can be studied specifically and in detail. Such approaches will not only increase our understanding about pathogenesis, but will also facilitate the development and introduction of new therapeutic strategies.

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**critical review**

# Anti-Inflammatory Therapy for Acute Lung Injury*

## A Review of Animal and Clinical Studies

*Craig Metz, Ph.D.;† and William J. Sibbald, M.D., F.C.C.P.*

**The adult respiratory distress syndrome (ARDS) continues to demonstrate high mortality. This syndrome is frequently observed as a remote complication of another disease process and is characterized by a significant inflammatory component. The purpose of this review is to compare and contrast published research on the use of anti-inflammatory agents, steroidal and nonsteroidal, in animal models of acute lung injury. Emphasis is given to the nature of the experimental pulmonary injury, infusion (*ie*, oleic acid and zymosan-activated plasma) or bacteriologically (*ie*, endotoxin and live bacteria) induced and the timing of drug administration relative to induction of the insult. The clinical data available on the use of these drugs in ARDS are discussed, and a rationale is presented for future clinical trials in these patients.**

*(Chest 1991; 100:1110-19)*

**ALI = acute lung injury; EVLW = extravascular lung water; I-HSA = iodinated human serum albumin; (L/P)TP = lymph/plasma total protein ratio; NSAI = nonsteroidal anti-inflammatory; $P_{MV}$ = pulmonary microvascular hydrostatic pressure; $P_{PA}$ = pulmonary artery pressure; PVR = pulmonary vascular resistance; $Q_L$ = pulmonary lymph flow; $Q_{O_2}$ = oxygen delivery; $\dot{Q}s/\dot{Q}t$ = intrapulmonary shunt fraction; ROS = reactive oxygen species**

Effective management of the adult respiratory distress syndrome (ARDS) and its sequelae has remained problematic since this syndrome was first described in 1967.[1] Despite advancement in understanding the pathophysiology of ARDS and improved life support of patients with this form of acute respiratory failure, mortality of 50 to 90 percent remains common in published reports.[2-4]

A wide variety of animal models have been developed to explore both the pulmonary and nonpulmonary sequelae of acute lung injury (ALI). Both direct (*eg*, aspiration) and indirect (*eg*, sepsis, drug overdose) insults to the alveolocapillary membrane have been used in various animal species to model the clinical syndrome. Common to a majority of these models has been the description of a diffuse inflammatory reaction in the lung's microvasculature. The recognition that neutrophils, macrophages, and other components of the inflammatory cascade participate in the progression of ALI has resulted in the use of anti-inflammatory agents as pharmacologic probes to further define the pathophysiology of this syndrome. In fact, recent data from animal studies of anti-inflammatory therapy in ALI have generated interest in clinical trials of anti-inflammatory therapy for ARDS. In this review, it is our purpose to provide a comprehensive discussion of both animal and clinical data relative to the use of four anti-inflammatory agents in ALI (ibuprofen, indomethacin, meclofenamate, and methylprednisolone). We will conclude by briefly discussing the mechanisms by which anti-inflammatory therapy might modify the course of ALI.

In order to standardize this review and thereby allow for comparison between different treatments and models, all studies reviewed have been categorized according to the time that anti-inflammatory therapy was begun in relation to the mechanism responsible for the diffuse lung injury process (*ie*, before or after insult). The type of insult used to induce the ALI process has been further categorized into infusion-induced injury (*ie*, oleic acid, zymosan-activated plasma, and thrombin) vs bacteriologically induced injury (*ie*, endotoxin and live organisms). Finally, the pulmonary sequelae of the injury induced in untreated animals by the various processes reviewed have been summarized in the control column of Table 1.[5-43] The potential efficacy of each of the four drugs reviewed was examined relative to its ability to ameliorate ALI produced by these various insults. A summary of their respective activities and a comparison of the relationship between the timing of events in animal models of ALI and clinical ARDS is presented in Figure 1.

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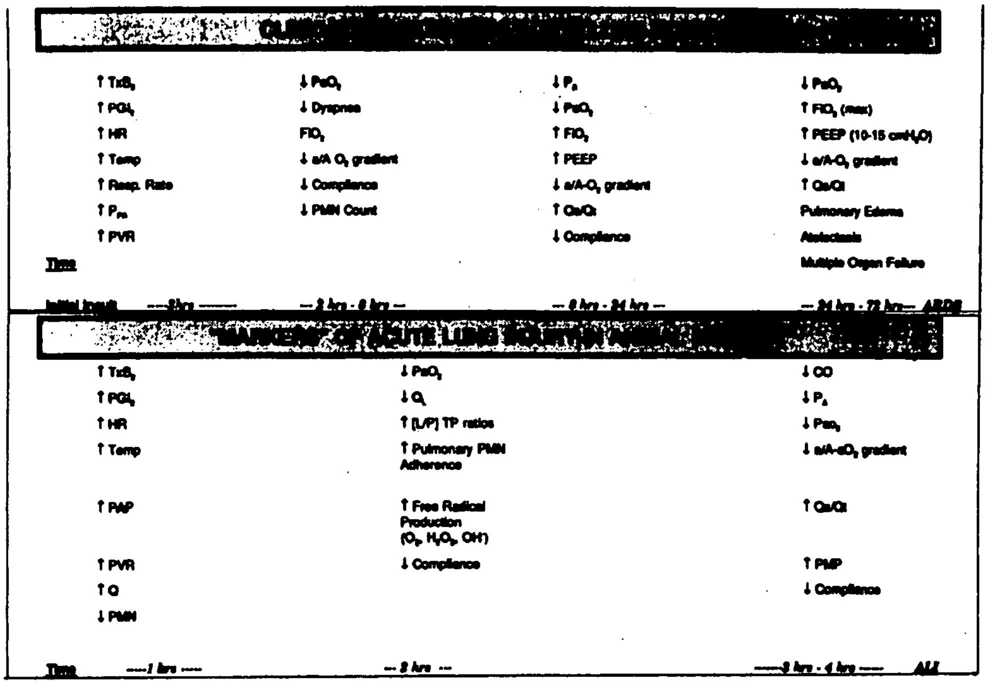
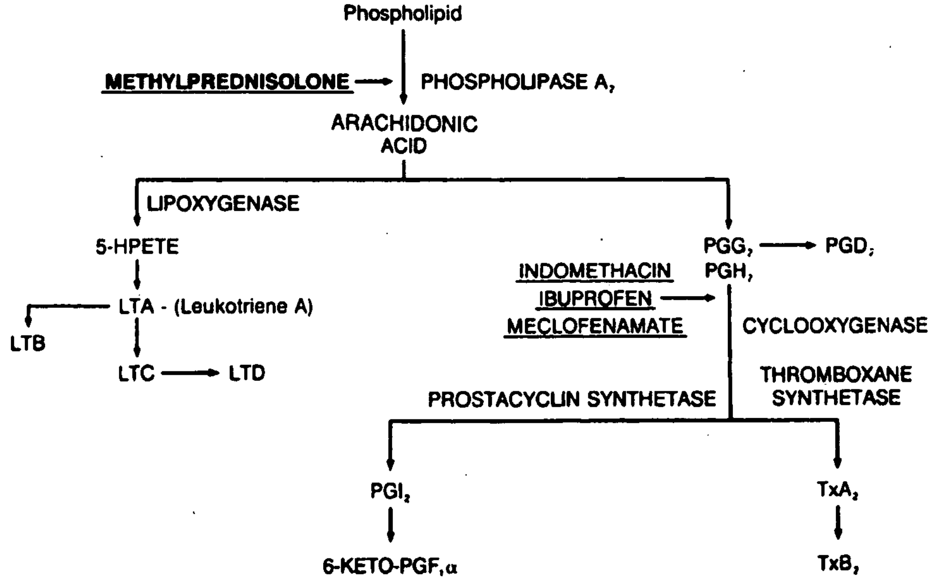


***RELATIVE EFFICACY OF ANTI-INFLAMMATORY AGENTS (ANIMAL MODELS)***

| | | | |
|---|---|---|---|
| Ibuprofen | ++++ | ++++ | ++ |
| Indomethacin | ++++ | +++ | + |
| Meclofenamate | ++ | + | - |
| MPSS | + | +++ | + |

FIGURE 1. Major features in progression of animal and clinical pulmonary injury. $TxB_2$, Thromboxane $B_2$; $PGI_2$, prostaglandin $I_2$; HR, heart rate; $FIo_2$, inspired oxygen tension; $A/AO_2$ gradient, alveolar-arterial oxygen tension gradient; PMN, polymorphonuclear neutrophil Pa, mean arterial perfusing pressures; PEEP, positive end-expiratory pressure; LP/TP, lymph-to-plasma total protein ratios; CO, cardiac output; PMP, pulmonary microvascular permeability; and MPSS, methyprednisolone sodium succinate.



Arachidonic Acid Cascade

FIGURE 2. Diagrammatic representation of arachidonic acid cascade, demonstrating where various anti-inflammatory agents discussed would modify production of vasoconstrictor and vasodilator prostaglandins.

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Table 1—*Animal Studies**

| Parameter | Control (Untreated) | Ibuprofen |  | Indomethacin |  | Meclofenamate |  | Methylprednisolone |  |
|---|---|---|---|---|---|---|---|---|---|
|  |  | Pre | Post | Pre | Post | Pre | Post | Pre | Post |
| Cyclooxygenase product | ↑ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | NC | NC |
| Blood pressure | ↑ ↓ | ↑ | NC | . . . | . . . | . . . | . . . | . . . | . . . |
| Cardiac output | ↓ | ↑ | ↑ | . . . | . . . | . . . | ↑ | . . . | . . . |
| LVSWI | ↓ | NC | . . . | . . . | . . . | . . . | . . . | . . . | . . . |
| $PaO_2$ | ↓ | ↑ | ↑ | ↑ ↓ | ↑ | ↑ | . . . | ↑ ↓ | ↑ |
| $\dot{Q}s/\dot{Q}t$ | ↑ | NC | ↓ | . . . | . . . | . . . | . . . | . . . | . . . |
| PPA | ↑ | ↓ | ↓ | ↓ | ↓ | NC | ↓ | ↑ ↓ | ↑ |
| SVR | ↑ | . . . | NC | . . . | . . . | . . . | . . . | . . . | . . . |
| PVR | ↑ | ↓ | ↓ | ↓ | ↓ | NC | ↓ | ↓ | . . . |
| EVLW | ↑ | . . . | ↓ | . . . | . . . | . . . | . . . | ↑ ↓ | ↑ ↓ |
| $\dot{Q}_L$ | ↑ | ↓ | ↓ | ↑ ↓ | . . . | ↑ ↓ | . . . | ↓ | . . . |
| (L/P)TP | ↑ | ↓ | ↓ | ↓ | . . . | ↑ ↓ | . . . | . . . | . . . |
| PMN count | ↓ | . . . | ↑ | ↑ | ↑ | ↑ ↓ | . . . | NC | . . . |
| PMN migration | ↑ | ↓ | . . . | . . . | . . . | ↓ | . . . | NC | . . . |
| PMN adherence | ↑ | ↓ | . . . | . . . | . . . | ↓ | . . . | . . . | . . . |
| References | . . . | 5-29 | 9,15,23, 24,30-36 | 10,12,18, 37,38 | 39 | 6,7,9,12,27, 29,40,41 | 36 | 18,25,26,39, 40,42,43 | 34,39 |

*NC, No change from untreated animals; LVSWI, left ventricular stroke work index; SVR, systemic vascular resistance; and PMN, polymorphonuclear neutrophil. Ellipses indicate data not reported.

## SPECIFIC ANTI-INFLAMMATORY AGENTS

### *Ibuprofen*

Ibuprofen is a derivative of propionic acid which acts as a cyclooxygenase inhibitor and thereby blocks production of both prostaglandin and thromboxane (Fig 2). Ibuprofen is commercially available in tablet form, although a parenteral formulation is under development and is available for investigational use.

*Animal Research: Infusion-Induced Injury.* Eleven studies of an infusion-induced pulmonary injury and pretreatment with ibuprofen were reviewed. At doses ranging from 6.25 to 25 mg/kg, pretreatment with ibuprofen inhibited the formation of both the stable metabolite of thromboxane $A_2$ and prostaglandin $I_2$.[5,9,11,12,27] Generally, the systemic and pulmonary circulatory responses to injury were simultaneously attenuated by ibuprofen, as anticipated increases in both mean pulmonary artery pressures (PPA)[7,9,10,27] and the calculated pulmonary vascular resistance (PVR)[7,9,27,28] were consistently blunted. Furthermore, when microspheres were used to induce ALI in rabbits, ibuprofen prevented a depression in cardiac output which was noted in an untreated group.[8] Whether this effect to "protect" against a depression in cardiac performance was a direct (*eg*, preventing abnormal ventricular interaction) or indirect (*eg*, preventing the release of myocardial depressant substance) process was not explored in this study.

Pretreatment with ibuprofen has also modified direct effects of ALI in the pulmonary microcirculation, as well as sequelae of the insult on arterial oxygenation. Thus, a depression in $PaO_2$ which followed the induction of ALI has been substantially prevented by pretreatment with ibuprofen.[9,26,28] Following ibuprofen pretreatment in large animals, changes in indices of altered microvascular fluid flux, *eg*, pulmonary lymph flow ($\dot{Q}_L$) and lymph/plasma total protein ratio ([L/P]TP), have been interpreted as demonstrating an effect of this anti-inflammatory drug to impart significant protection from the development of increased pulmonary microvascular permeability.[6,7,9,12,27] In a study of ALI secondary to thrombin infusion, Johnson and Malik[12] reported that anticipated increases in both pulmonary $\dot{Q}_L$ and (L/P)TP ratios were delayed by a 6.25-mg/kg loading dose; a 25-mg/kg dose completely prevented the pulmonary microvascular injury which normally results in augmented formation of extravascular lung water (EVLW) at normal hydrostatic pressures in ALI.[12] Finally, pretreatment with ibuprofen reduced neutrophil adherence to both skin[11] and pulmonary endothelium[27] following the administration of chemotactic agents such as leukotriene $B_4$, zymosan-activated plasma, or thrombin. A concentration-dependent reduction in neutrophil superoxide anion production was also demonstrated following ibuprofen administration.[27]

Two studies were reviewed which described infusion-induced ALI models and post-treatment with ibuprofen. A study by Hammond et al[10] demonstrated the same general pattern of protection which was reported in the previously reviewed studies of pretreatment. Specifically, ibuprofen prevented anticipated increases in all of the mean PPA, $\dot{Q}_L$, and lung wet/dry weight ratios which otherwise followed the infusion of sclerosing agents. A second study employed a plant toxin to induce ALI in rats; and, like others, this model was characterized by an increase in pulmonary microvascular permeability, pulmonary hypertension, and the development of right ventricular hypertrophy;[32] however, unlike other infusion-induced models of ALI, the pulmonary lesion in this model developed five days after administration of the plant toxin. When compared with untreated control animals, 14 days of treatment with ibuprofen resulted in no significant decrease of either lung wet/dry weight ratios or right ventricular hypertrophy.[32]

*Animal Research: Bacteriologically Induced Injury.* Of the 19 studies reviewed which reported a bacteriologically induced insult, 13 used live *Escherichia coli* or *E coli* endotoxin,[13-22,25,30,44] four used live *Pseudomonas aeruginosa* infusion in pigs[23,33,34] or sheep,[35] one used group B Streptococcus in pigs,[24] and two others induced peritonitis in sheep by either perforating the cecum[31] or by infusing live *E coli* into the peritoneal cavity.[15] In the subsequent discussion of these latter data, distinction will be made between what has been described as the phase 1 and phase 2 responses of ALI which typically follow the infusion of both endotoxin and live bacteria in sequence. During the phase 1 response, an increase in pulmonary $\dot{Q}$L is thought to be primarily the result of an abrupt increase in microvascular hydrostatic pressure (Pmv) complicating acute pulmonary hypertension. During the phase 2 response the mean PPA falls, yet both pulmonary $\dot{Q}$L and (L/P)TP ratios remain elevated or increase further. This phase 2 response describes a time in the progression of ALI which is considered to represent an increase in pulmonary microvascular permeability.

Generally, the effects of ibuprofen pretreatment in bacteriologically induced ALI were most impressive during the acute, or phase 1, response to ALI.[20] During this stage an increase in vasoconstrictor prostanoids (*eg*, thromboxane $A_2$) is thought to contribute to both the immediate pulmonary hypertensive response and the accompanying ventilation-perfusion mismatch which underlies arterial hypoxia in animal models of ALI. As reported in infusion-injury models, pretreatment with ibuprofen in these studies of bacteriologically induced ALI attenuated both the injury-related arterial hypoxia[15,16,18,21,23,30] and acute pulmonary hypertension.‡ Thus, the phase 1-mediated decrease in $PaO_2$ and increase in mean PPA have been either prevented or substantially ameliorated by ibuprofen pretreatment in ALI.

In studies where pulmonary microvascular fluid flux was simultaneously assessed by measuring changes in lung $\dot{Q}$L, ibuprofen pretreatment also significantly modified expression of the disease. Extension of benefit into the permeability, or phase 2, response of ALI was demonstrated when treatments with ibuprofen and diphenhydramine were combined.[20] Both *in vitro* neutrophil adherence[21] and the degree of neutrophil accumulation in bronchoalveolar lavage fluid[14,25] have been significantly reduced with ibuprofen pretreatment. As with infusion-induced ALI models, a dose-dependent effect on neutrophil function was probable, since low doses of ibuprofen enhanced neutrophil function,[14,25] while higher doses had an inhibitory effect.[14,21,25]

Compared to pretreatment protocols, studies which have employed a postinsult treatment regimen[15,23,24,30,31,33-36] have not resulted in the same magnitude of protection from the sequelae of ALI; however, cyclooxygenase inhibition has been demonstrated with all doses of ibuprofen used in these studies. Some amelioration of the anticipated increase in mean PPA accompanied postinsult treatments when ibuprofen was combined with either prostaglandin $E_1$[33] or methylprednisolone.[34] Other than an improvement in the stroke volume index, the combination of ibuprofen with prostaglandin $E_1$ did not provide additive benefit, although survival time was significantly extended by ibuprofen in the latter study.[33]

*Clinical Trials.* A parenteral formulation of ibuprofen has not been available for widespread clinical testing; however, two clinical reports of experience with oral ibuprofen (Motrin) may be relevant to understanding the potential effects of this drug in ARDS. When normal volunteers were rendered hypoxic by breathing a mixture of 12.5 percent oxygen in nitrogen, the $PaO_2$ fell to below 50 mm Hg while the PVR concurrently rose.[45] Although pretreatment with ibuprofen in this study was accompanied by a reduction in serum thromboxane $B_2$ levels by 85 percent, there was no associated short-term effect on arterial hypoxemia. The second clinical report was a case study of a patient with ARDS[46] who manifested significant pulmonary hypertension, hypoxemia, and radiographic evidence of diffuse pulmonary infiltrates. Treatment with a 400-mg oral dose of ibuprofen was followed by the infusion of an ibuprofen solution at the rate of 50 mg/h for 9 h and 30 mg/h for an additional 15 h. Within 4 h of starting treatment, the $PaO_2$ rose, while the mean PPA fell. An inadvertent increase in dosage resulted in a further decrease in the mean PPA, while termination of therapy was associated with an abrupt increase in the mean PPA.[46]

Two *in vitro* studies which studied chemotactic stimulation of human neutrophils were also reported.[47,48] Ibuprofen inhibited neutrophil aggregation and migration, enzyme release, and cell swelling but did not depress neutrophil integrity or bactericidal activity. As with the animal studies reviewed, these drug effects were more pronounced with higher blood levels of ibuprofen.

### *Indomethacin*

Indomethacin is a derivative of indoleacetic acid with anti-inflammatory, antipyretic, analgesic, and cyclooxygenase-inhibitory properties. Indomethacin is marketed as an oral capsule. Although available in a parenteral formulation, this is restricted to approval for the closure of patent ductus arteriosus.

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‡References 15, 16, 18-20, 22, 23, 30, and 44.

*Animal Experiments: Infusion-induced Injury.* In infusion-induced ALI models,[10,12,37] the effects of pretreatment with indomethacin were generally similar to those previously noted with ibuprofen; for example, significant cyclooxygenase inhibition has been demonstrated at all doses tested. Following the infusion of sclerosing agents, pulmonary hypertension was prevented by pretreatment with indomethacin.[10] Increases in pulmonary $\dot{Q}_L$ during the early phase 1 response to thrombin-induced ALI were also blunted by indomethacin pretreatment, although this effect did not extend into phase 2 of the ALI process.[12] Results from the sole canine study which used zymosan-activated plasma to induce ALI[39] demonstrated that indomethacin was effective in preventing anticipated arterial hypoxemia, while it simultaneously minimized an abrupt increase in the mean PPA.

*Animal Research: Bacteriologically Induced Injury.* In infusion models of *E coli* endotoxin-induced pulmonary injury,[18,38] pretreatment with indomethacin at a 1.5-mg/kg loading dose prevented an elevation in the plasma levels of prostaglandins E and $F_2$,[18] while injury-induced pulmonary hypertension and acute hypoxemia were simultaneously prevented. Indomethacin enhanced the formation of leukotrienes in a rat model of neutrophil activation.[49] Indomethacin pretreatment also limited an expected increase in free oxygen radical production which accompanied cellular stimulation with phorbol myristate acetate.[49]

*Clinical Trials.* No trials of indomethacin therapy in patients with ARDS have been reported.

### *Meclofenamate*

Meclofenamate is a derivative of *o*-aminobenzoic acid (anthranilic acid) which possesses anti-inflammatory, analgesic, antipyretic, and cyclooxygenase inhibitory characteristics similar to the other nonsteroidal anti-inflammatory drugs which have been previously discussed. Meclofenamate is commercially available as an oral capsule.

*Animal Experiments: Infusion-Induced Injury.* Six studies which employed meclofenamate in infusion-induced models of ARDS were reviewed, and all were pretreatment protocols.[6,7,9,12,27,29] Doses in the range of 3 to 5 mg/kg were effective in inhibiting the production of thromboxane $B_2$[6,7,9,12,27] and 6-keto-PGF$_1\alpha$.[6,12,27] Generally, the effect of meclofenamate during both the initial (*ie*, phase 1) and steady-state permeability (*ie*, phase 2) stages of ALI was less impressive than has been reported with indomethacin or ibuprofen pretreatment.

Pretreatment with meclofenamate prevented the decrease in $PaO_2$ which otherwise accompanied an infusion of zymosan-activated plasma.[9] Of all of the pretreatment protocols reviewed,[6-8,12,27] prevention of the abrupt phase 1 increase in the PPA was evident in only one study.[7] Prevention of an increase in either pulmonary $\dot{Q}_L$ or (L/P)TP ratios was not predictable when meclofenamate was used in various ALI models. When meclofenamate influenced pulmonary $\dot{Q}_L$, effects were usually confined to the initial or phase 1 stages in the development of ALI. The presence of leukopenia, perhaps implying neutrophil sequestration in the lung's microvasculature, was unaffected by treatment with meclofenamate. Meclofenamate's effect on neutrophil migration and adherence was less than was demonstrated with ibuprofen.[27] Although meclofenamate had a significant effect on superoxide anion production, this effect was only achieved at supraphysiologic drug concentrations.[27]

*Animal Research: Bacteriologically Induced Injury.* In two studies which employed *E coli* endotoxin infusion in sepsis,[40,41] meclofenamate pretreatment prevented the early increase in both mean PPA and $\dot{Q}_L$, as well as the simultaneous hypoxemia. The combination of methylprednisolone and meclofenamate in one of these studies[40] attenuated most of the phase 1 as well as the phase 2 effects of an *E coli* infusion. In rabbits undergoing microsphere-induced pulmonary embolization, pretreatment with meclofenamate prevented an increase in the PPA as well as the fall in both cardiac output and stroke volume, while systemic oxygen consumption was significantly decreased.[29] In the single study reporting a post-insult treatment, Gregory et al[36] examined the effects of meclofenamate on hypoxic pulmonary vasoconstriction in an isolated ferret lung model and found this anti-inflammatory drug was ineffective in preventing hypoxic pulmonary vasoconstriction.

*Clinical Trials.* No clinical trials of meclofenamate treatment in ARDS were found.

### *Methylprednisolone*

Methylprednisolone is a synthetically produced corticosteroid which possesses potent anti-inflammatory activity. It inhibits synthesis of products of the arachidonic acid cascade by blocking the action of phospholipase $A_2$ (Fig 2).

*Animal Research: Infusion-Induced Injury.* An infusion-induced injury was used in two canine models of ALI.[26,39] Pretreatment with methylprednisolone prevented an expected increase in the mean PPA but did not affect the leukopenia resulting from the infusion of zymosan-activated plasma in these studies. Increases in EVLW have not been prevented by methylprednisolone.[26] Even though the dose of methylprednisolone used (30 mg/kg) should have been sufficient to inhibit the arachidonic acid cascade, there was no noticeable reduction in thromboxane $B_2$ in the one study where it was measured.[26]

*Animal Research: Bacteriologically Induced Injury.* In pretreatment studies using an *E coli* endotoxin-induced ALI, the phase 1 increase in PPA was generally unaffected by methylprednisolone in the treated sheep,[18,43] and a consistent benefit of steroids on oxygenation was not reported. With methylprednisolone, the mean $PaO_2$ was increased over the control group in two studies[40,42] but was not different from that found in untreated animals in two other studies.[18,43] Phase 2 vs phase 1 increases in pulmonary $\dot{Q}L$ and EVLW have been modestly attenuated by methylprednisolone.[40,42,43]

Both endotoxin-induced leukopenia[40,43] and increased pulmonary neutrophil migration[25] were unaffected by methylprednisolone treatment. Demling et al[43] reported that methylprednisolone attenuated the plasma increases in both β-glucuronidase and arylsulfatase subsequent to endotoxin administration while also reducing lysosomal enzyme activity in lung lymph.[36]

A post-insult treatment protocol was used in two porcine models of ALI, and the results of these studies are contradictory. In one study,[42] methylprednisolone treatment was associated with an improved $PaO_2$ and prevented the dramatic increase in EVLW noted in the control animals. In another study,[34] methylprednisolone had no effect on the mean PPA, EVLW, or stroke volume index and demonstrated only a slight inhibitory effect on the generation of cyclooxygenase metabolites.

When methylprednisolone has been used alone, it is interesting to note that its effects are reportedly more pronounced in the permeability of phase 2 stage of endotoxin-induced ALI. When methylprednisolone was combined with a nonsteroidal anti-inflammatory agent, *ie*, meclofenamate,[40] both the phase 1 and phase 2 changes which characterize ALI secondary to an endotoxin infusion were attenuated.

*Clinical Trials.* Based on results of animal research, methylprednisolone was subjected to clinical trials as adjunctive therapy for patients with ARDS. Sibbald et al[50] used the clearance of iodinated human serum albumin (I-HSA) to demonstrate increased pulmonary capillary permeability in 19 patients with ARDS. This study reported that treatment with methylprednisolone at 30 mg/kg was associated with a significant reduction in I-HSA clearance. Responders and nonresponders to methylprednisolone were identified on the basis of the mean PPA and $\dot{Q}s/\dot{Q}t$ at admission to the study, with a lower mean PPA and $\dot{Q}s/\dot{Q}t$ characterizing the steroid responders. Finally, mortality was 21 percent (3/14) in the responders vs 100 percent (5/5) in the nonresponders.

Two prospective clinical trials subsequently evaluated methylprednisolone as prophylaxis against ARDS in high-risk trauma patients. In one study, it was suggested that methylprednisolone-treated patients with injury scores less than 50 developed ARDS at a significantly lower rate than control patients with similar scores.[51] Overall, ARDS occurred in significantly fewer methylprednisolone-treated patients (3/47 with methylprednisolone vs 11/45 controls; $p<0.05$). These results were in contrast to another prospective study in which a high-risk population of surgical patients was identified on the basis of blood gas criteria.[52] In this latter study, ARDS developed in significantly more methylprednisolone-treated patients (methylprednisolone, 25/49 [51 percent], placebo, 14/42 [33 percent]; $p<0.008$), and no significant differences in mortality were observed between these two groups.

The recent publication of three randomized, double-blind, placebo-controlled clinical trials of methylprednisolone in patients with either established ARDS or at risk of developing ARDS has cast significant doubt on its efficacy in these clinical situations.[53-55] In the study by Bernard et al,[53] a four-dose 30-mg/kg regimen of methylprednisolone had no effect on oxygenation, hemodynamics, ARDS reversal, or mortality. Bone et al[54] also reported that a similar regimen of methylprednisolone was ineffective in either preventing ARDS development or in increasing its rate of reversal. As with studies by Bernard et al[53] and Bone et al,[54] the results of a clinical trial by Luce and colleagues[55] failed to demonstrate any benefit of methylprednisolone on ARDS development or subsequent death.

## DISCUSSION

We have previously reviewed the complex pathophysiology of ARDS[56] and noted that mortality from this form of acute respiratory failure was essentially unaltered from that reported when this syndrome was described over 20 years ago. Nonetheless, it is generally accepted that the spectrum of physiologic abnormalities recognized as comprising this syndrome has changed. Thus, it is now generally accepted that patients rarely die from the direct sequelae of ARDS, including arterial hypoxemia. More frequently, mortality usually follows a protracted clinical course which culminates in the dysfunction or failure of both the lung and extrapulmonary organs, *ie*, multiple organ failure. Other changes have also been noted in the profile of patients developing ARDS; for example, patients with ARDS now tend to reflect an older and more complex case scenario than noted previously, most likely due to improvements in resuscitation in medical and surgical emergencies.

Improved understanding of the multiple processes which interact to result in ARDS have been advanced, in part, through the development of clinically applicable animal models. A number of common themes have emerged from research on ALI in these varied models. In particular, it is now generally accepted that an important interaction between both the process of inflammation and the products of the arachidonic acid cascade with the lung's microcirculation are likely crucial for the full expression of ALI. As a result of the notion that an unchecked inflammatory response within the pulmonary microcirculation characterizes a majority of the most relevant animal models of ALI, a number of studies have been carried out to explore the hypothesis that nonsteroidal anti-inflammatory drugs (NSAIs) would ameliorate the course of specific components of the sequence of events resulting in ALI. Therefore, while a number of the studies herein reviewed provide information as to the mechanism of injury, we also found that post-treatment studies, albeit few in number, were undertaken to specifically address the hypothesis that NSAIs might ameliorate the progression of ALI, once initiated.

This review of reported studies employing anti-inflammatory therapy in various models of ALI demonstrate an important and positive interaction between therapy with this class of drug and disease progression. Specifically, some anti-inflammatory agents have been shown to substantially modify the course of pulmonary hypertension, arterial hypoxemia, and specific components of the inflammatory cascade during the progression of ALI.

### *Anti-inflammatory Therapy and Pulmonary Hypertension*

Cyclooxygenase inhibition was demonstrated with each of the anti-inflammatory drugs reviewed. Blocking an increase in plasma levels of the powerful vasoconstrictor, thromboxane $A_2$, with ibuprofen was associated with the blunting of an anticipated increase in mean $P_{PA}$, regardless of this NSAI's administration in a pretreatment or post-treatment protocol. Importantly, this effect to ameliorate the pulmonary hypertensive response of ARDS was accomplished without further depressing $SaO_2$. In the long term, it might be hypothesized that blunting an increase in mean $P_{PA}$ should reduce the degree of EVLW accumulation in ARDS, as transmittal of an elevated $P_{PA}$ to the lung's fluid-exchanging elements increases the hydrostatic pressures and thereby contributes to increased fluid egress across the lung microvascular barrier membrane. Furthermore, preventing the pulmonary hypertensive component of ALI should also contribute to improved myocardial performance in this illness, as acute pulmonary hypertension may depress left ventricular performance through a number of different mechanisms. This latter issue may be why improved systemic flows were reported in some post-treatment studies with ibuprofen and meclofenamate.

### *Anti-inflammatory Therapy and Arterial Hypoxemia*

In both pretreatment and post-treatment protocols using anti-inflammatory therapy, the magnitude of hypoxemia accompanying an ALI insult was not as severe as described in companion study groups not receiving this treatment. Arterial hypoxemia in ARDS results from multiple etiologies. In endotoxin models of ALI, an acute depression in the $SaO_2$ occurs before any significant increase in EVLW and concurrent with an abrupt increase in the mean $P_{PA}$. This process presumably reflects the effect of acute pulmonary hypertension to modify normal $\dot{V}_A/\dot{Q}$ relationships in the lung independent of acute changes in pulmonary microvascular fluid flux.[40] With the progression of ALI, an increase in EVLW can eventually be correlated with a depression in the $SaO_2$, presumably by the effect of peribronchial edema and alveolar collapse to increase shunted lung units. Therefore, potential reasons for improved oxygenation in ALI treated with NSAIs might include (1) amelioration of a pulmonary hypertension, thereby restoring more physiologic $\dot{V}_A/\dot{Q}$ relationship, and (2) a later reduction in EVLW accumulation, thereby also improving upon altered $\dot{V}_A/\dot{Q}$ relationships.

### *Anti-inflammatory Therapies and the Neutrophil in ARDS*

It has been demonstrated that the accumulation of neutrophils in the lung's microcirculation may be an important precipitating event in the development of increased pulmonary microvascular permeability in animal models of ARDS, as well as in the clinical setting. In animal studies, treatment with ibuprofen or indomethacin attenuated the depressed neutrophil count observed in control groups, while meclofenamate did not exhibit this effect. Neutrophil migration and adherence were reduced during pretreatment with ibuprofen or meclofenamate but were not reported for indomethacin or any of the postinsult treatment studies reviewed. If the hypothesis regarding the relationship between pulmonary neutrophil entrapment and a subsequent increase in the lung's microvascular permeability is correct,[11] then an increase in EVLW is likely to occur to a greater extent in control vs the NSAI-treated experimental groups. Thus, both pretreatment and post-treatment with ibuprofen were associated with a signifigant amelioration in all of the $\dot{Q}_L$, EVLW, and (L/P)TP ratios, while meclofenamate had the weakest positive effect at this level.

One of the proposed mechanisms by which neutrophils cause ALI is through the production and extracellular elaboration of toxic reactive oxygen species (ROSs). These compounds have the ability to induce lipid peroxidation of cell membranes, leading to an increased prostanoid production and increased microvascular permeability. Although the mechanism of effect may be debatable, benefits of anti-inflammatory drugs may have been related to their effect on excessive ROS-induced tissue injury. Ibuprofen was found to have a suppressive effect on superoxide anion production, while indomethacin actually caused a moderate increase in production of this oxidant.

### *NSAIs and Adverse Sequelae*

The primary focus of animal research with nonsteroidal agents in ALI models has been on the evaluation of efficacy; however, the potential for adverse effects following the use of these drugs in the clinical setting cannot be ignored. Clive and Stoff[57] drew attention to the possibility of detrimental effects on the kidney following prostaglandin inhibition with anti-inflammatory therapy by NSAIs. Generally, studies reporting a benefit on ALI in various animal models did not discuss any adverse effects of the NSAIs in detail.

### *Methylprednisolone and ALI*

In contrast to the effect of NSAIs, only weak cyclooxygenase inhibition was observed with methylprednisolone. Since early changes in pulmonary hemodynamics are thought to be related to the elaboration of vasoconstrictive thromboxane products, it was not surprising that acute increases in the mean PPA and PVR were not consistently benefited by administration of this corticosteroid.

The major reproducible component of steroid efficacy in lung injury models was evident only in the later stages of development of the pulmonary injury, where indicators of increased microvascular permeability, EVLW, and $\dot{Q}L$ were favorably affected by corticosteroid pretreatment, but not by post-treatment; however, recent prospective, double-blind clinical trials of patients with ARDS or at risk of developing ARDS have failed to demonstrate any significant, beneficial steroidal effect. Reports of an increased incidence of infectious complications[52,58,59] and the adverse association of steroid therapy and renal insufficiency[59] further negate consideration of corticosteroid treatment for this troup of patients.

## SUMMARY

We are of the opinion that existing animal data support a proposal for a clinical trial of NSAI agents in ARDS. In many animal models, this review found evidence for consistent benefit of nonsteroidal agents on the progression of ALI. We speculate that both improved arterial oxygenation and systemic flows may be demonstrated with NASIs in a clinical trial. The net effect of an improved $PaO_2$ and $SaO_2$, together with an increase in systemic flows, should be an increase in systemic oxygen delivery ($Qo_2$). Were nonsteroidal agents to augment systemic $Qo_2$, mortality might be reduced, since a covert oxygen debt complicates ARDS;[60] and studies by Shoemaker et al,[61] Bihari et al,[60] and others[62] have directly or indirectly correlated outcome from sepsis and ALI with evidence for maintaining a "supranormal" systemic $Qo_2$; however, augmenting systemic $Qo_2$ may not necessarily improve tissue oxygen availability, and we found few studies which had critically analyzed the effect of NSAIs on tissue oxygen availability or other extrapulmonary markers of organ function. Therefore, although it might be anticipated that improvement in the pulmonary lesion of ARDS might accompany treatment with NSAIs, there are insufficient data to predict their effect on the extrapulmonary organ sequelae of ARDS which may herald the onset of multiple system organ failure.

Finally, clinical trials with these compounds should include careful monitoring of renal function, prostaglandin production, and circulating blood levels of the drug in order to determine the effect of acute administration of these drugs to critically ill patients. If nonsteroidal agents are found to have an adverse effect on renal function, it may be possible to ameliorate the effect by reducing blood drug levels or using concomitant therapy such as dopamine[64] to block the renal effect of these compounds.

ACKNOWLEDGMENTS: We thank Ms. Lesley Ross, Ms. Carol Buckhout, and Ms. Cathy Carvell for secretarial assistance in preparing this manuscript.

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